An Indirect ELISA Method for the Quantitative Determination of 3,5,6-Trichloro-2-pyridinol (TCP) in Urine

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1 ABSTRACT

A sensitive, competitive enzyme-linked immunosorbent assay (ELISA) for 3,5,6-trichloro-2-pyridinol (TCP) has been developed to quantitate parts per billion (ppb) amounts of the analyte in urine. The assay uses a hapten-protein conjugate that is coated on to a 96-well microtiter plate and a monoclonal antibody. TCP is a major metabolite and environmental degradation product of the insecticide chlorpyrifos and the herbicide triclopyr. For these reasons, TCP can serve as a biomarker for monitoring levels of exposure or contamination for these pesticides. The level of detection (LOD) estimated for this assay is 0.01 ppb in hydrolyzed and extracted urine samples, diluted in assay buffer. Immunoassay results compared very well with GC/MS measurements (r = 0.970) for samples obtained from 2 exposure monitoring studies.

2 INTRODUCTION

The Human Exposure Research Branch of the U.S. Environmental Protection Agency - Las Vegas, NV, through its immunochemistry program, has been developing methods for monitoring pesticide residues. Because clorpyrifos (O,O-diethyl-O-[3,5,6-trichloro-2-pyridyl]-phosphorothioate) is an extensively used organophosphorous insecticide it is of major interest in many monitoring studies. Since the known biomarker of exposure for chlorpyrifos is 3,5,6-trichloro-2-pyridinol (TCP) we developed a fast, economical immunoassay to quantitate TCP in urine to assist exposure assessments in large monitoring studies. Current methods of detection for TCP using traditional analytical methods require specific and labor intensive sample preparation and testing which can become very costly for large sample groups. Using immunoassay as a screening tool, exposure assessment analyses can be done faster. The assay has a nine point standard curve (0.0, 0.098, 0.38, 0.78, 1.56, 3.13, 6.125, 12.5, 25.0 ng/mL) using an analysis volume of 100 µL.

Because TCP is found conjugated in urine, it is necessary to first hydrolyze and extract the samples. The extract is then layered on top of the assay buffer (phosphate buffered saline with tween, PBST) before evaporation by nitrogen and final dilution for the immunoassay. This sample preparation can easily be performed on sixty samples the day prior to analysis. Since the sensitivity of the immunoassay is at a low level (0.01 ng/mL), only 1 mL of urine is required. For GC/MS determination, sample splits can be removed after the hydrolysis and extraction steps. The sample for GC/MS is evaporated under a nitrogen stream, brought up to 1 mL in o-xylene and derivatized. This dual analysis is helpful for confirmation and quality assurance (QA). Using a 96-well microtiter plate, twenty-four samples in duplicate can be run with controls and one standard curve per plate. One person can assay three to six plates per day. When diluted, the complex urine matrix does not interfere in the assay. Therefore, standards can be prepared in PBST without the addition of a control urine.

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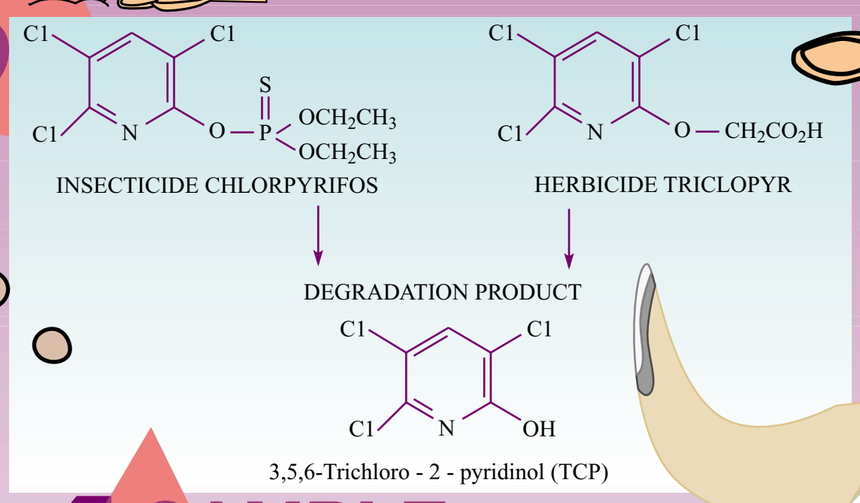


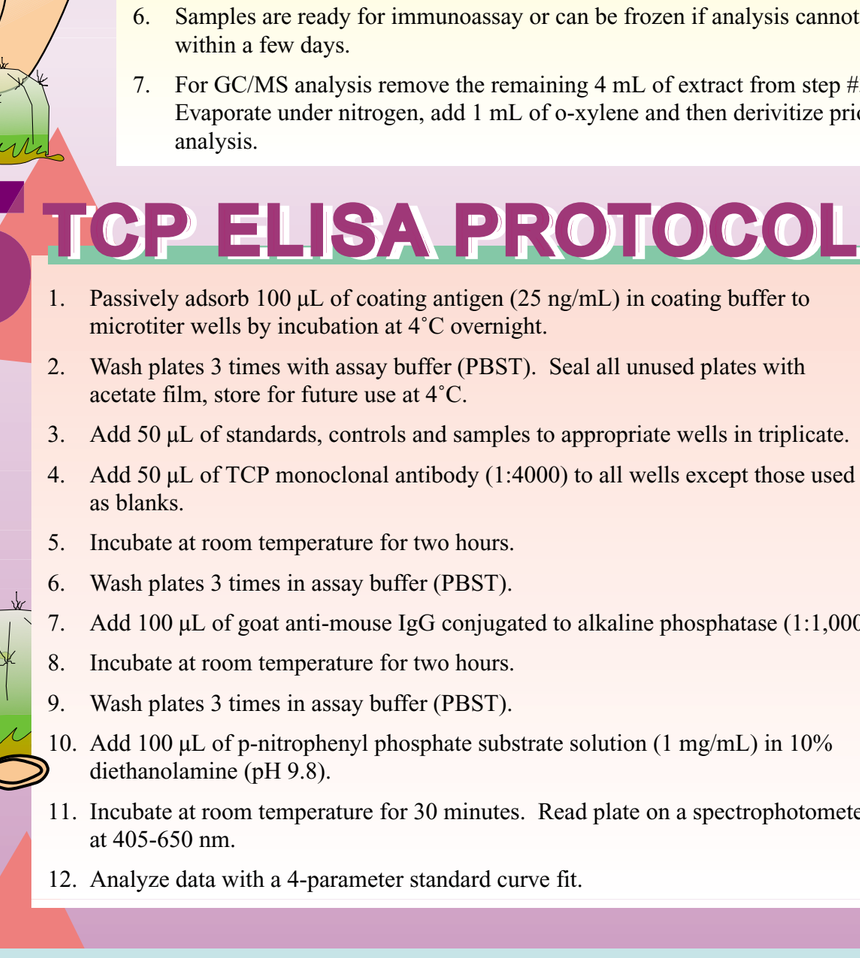
4 SAMPLE PREPARATION

1. Add 100 µL of conc. HCl to 1 mL of urine in a screw cap glass tube.
2. Hydrolyze for two hours at 80˚C, remove from heat, allow to cool (no cap).
3. Extract TCP from hydrolysate with 5 mL of 1-chlorobutane, cap tube, vortex well. Allow phases to separate.
4. Remove 1 mL of extract and layer on top of 2 mL of assay buffer (PBST) for the immunoassay.
5. Evaporate the organic layer under a gentle nitrogen stream.
6. Samples are ready for immunoassay or can be frozen if analysis cannot be done within a few days.
7. For GC/MS analysis remove the remaining 4 mL of extract from step #3. Evaporate under nitrogen, add 1 mL of o-xylene and then derivitize prior to analysis.

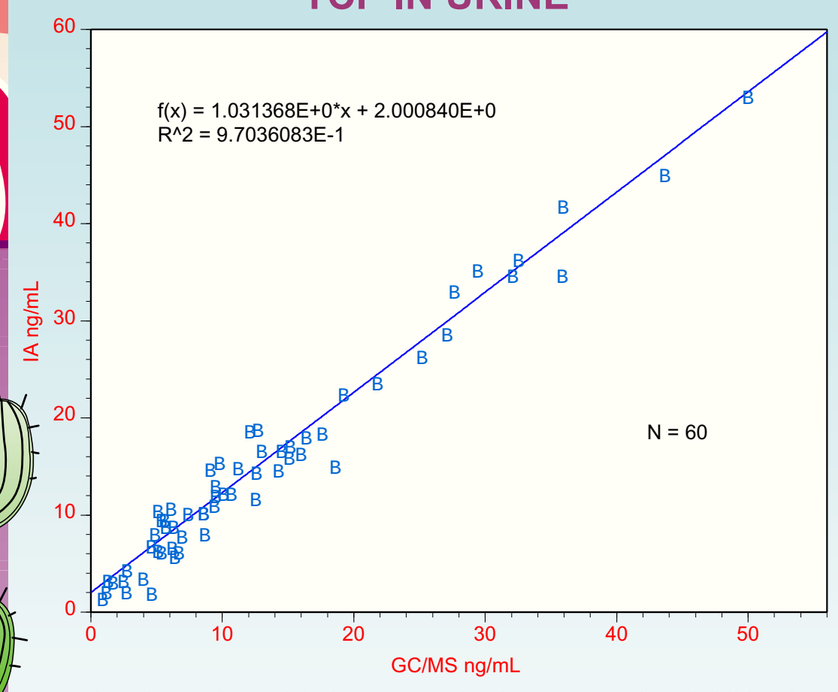
5 TCP ELISA PROTOCOL

1. Passively adsorb 100 µL of coating antigen (25 ng/mL) in coating buffer to microtiter wells by incubation at 4˚C overnight.
2. Wash plates 3 times with assay buffer (PBST). Seal all unused plates with acetate film, store for future use at 4˚C.
3. Add 50 µL of standards, controls and samples to appropriate wells in triplicate.
4. Add 50 µL of TCP monoclonal antibody (1:4000) to all wells except those used as blanks.
5. Incubate at room temperature for two hours.
6. Wash plates 3 times in assay buffer (PBST).
7. Add 100 µL of goat anti-mouse IgG conjugated to alkaline phosphatase (1:1,000).
8. Incubate at room temperature for two hours.
9. Wash plates 3 times in assay buffer (PBST).
10. Add 100 µL of p-nitrophenyl phosphate substrate solution (1 mg/mL) in 10% diethanolamine (pH 9.8).
11. Incubate at room temperature for 30 minutes. Read plate on a spectrophotometer at 405-650 nm.
12. Analyze data with a 4-parameter standard curve fit.

6 4-PARAMETER STANDARD CURVE FOR 3,5,6-TRICHLORO-2-PYRIDINOL (TCP)

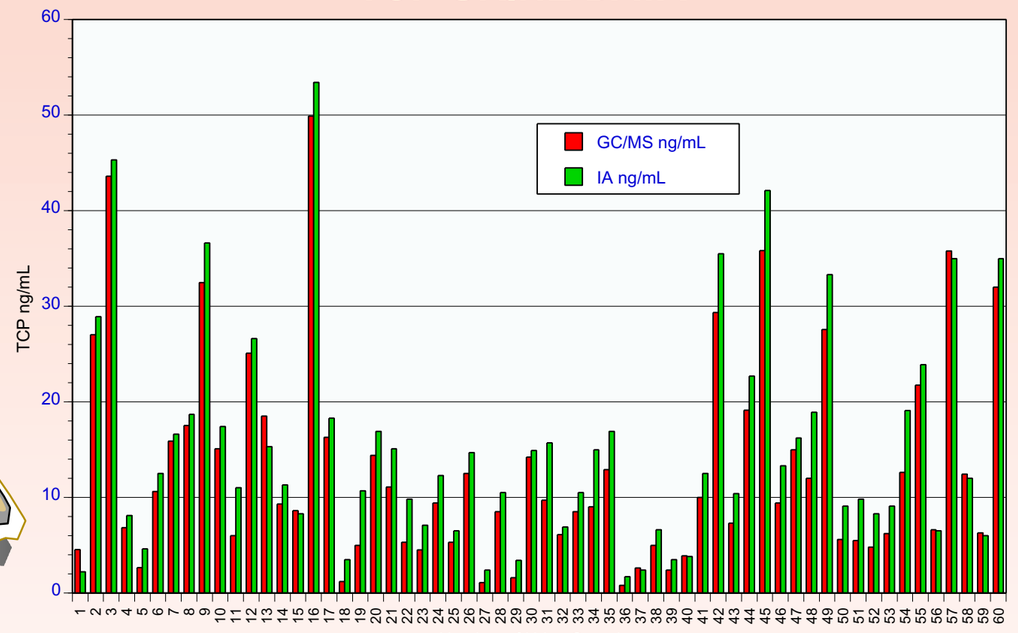


7 GC/MS vs. IMMUNOASSAY TCP IN URINE



Samples are from adults in an occupational exposure study and children in an exposure assessment group. Samples were analyzed by the indirect ELISA and electron impact GC/MS. Assays will be used to screen additional samples for determining exposure levels.

8 TCP URINE DATA



Graphic representation of TCP correlation data.

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QUALITY CONTROL DATA FOR TCP IMMUNOASSAY N=20

STATISTICS	INTER ASSAY CONTROL - 1	INTER ASSAY CONTROL - 2	INTRA ASSAY CONTROL - 1	INTRA ASSAY CONTROL - 2
MEAN	1.03	4.035	0.994	3.98
SD	0.05	0.024	0.07	0.267
CV%	4.9	6.0	7.2	6.7

Control 1 = 1.0 ng/mL TCP in PBST
Control 2 = 4.0 ng/mL TCP in PBST

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3,5,6-TRICHLORO-2-PYRIDINOL (TCP) RECOVERY OF SPIKES FROM URINE SAMPLES THAT WERE HYDROLYZED AND EXTRACTED WITH 1-CHLOROBUTANE THEN ANALYZED BY IMMUNOASSAY

SAMPLE POOL	ASSAY MEAN	SD	CV%	SPIKE ng/mL	CORRECTED FOR DILUTION	VALUE MINUS POOL	RELATIVE % RECOVERY
POOL	3.3	0.259	7.64	0.0	16.5	-	-
SPIKE 1	3.9	0.447	11.4	3.0	19.5	3.0	100.0
SPIKE 2	4.5	0.308	7.3	6.0	22.3	5.8	96.6
SPIKE 3	6.34	0.523	8.2	15.0	31.7	15.2	101.3
SPIKE 4	9.97	1.384	12.6	30.0	49.8	33.3	111.0
SPIKE 5	11.9	2.8	16.9	45.0	60.0	43.5	96.6

11 CONCLUSION

The performance of this method has been verified by many samples using EPA Methods set forth in SW-846 such as 8140, and 8141A, Gas Chromatography (GC) and Gas Chromatography/Mass Spectrometry (GC/MS). The immunoassay has been found to be accurate and precise and correlates well with traditional analytical methods.

In addition to using the TCP assay for human exposure monitoring, immunoassays for parent compounds could determine the extent of environmental contamination. The combination of these data would provide indepth exposure scenarios and risk assessments.

12 SPECIAL THANKS

We gratefully acknowledge J.J. Manclus and A. Montoya, Laboratorio Integrado Biogeniera, Universidad Politecinca de Valencia, Valencia, Spain, for the generous gift of TCP antibodies and conjugate which made these studies possible. We would also like to extend our thanks to Chuck Mihalak of Dow Elanco, Indianapolis, Indiana, for his guidance in the extraction protocol.